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Relation of intra-splenic migration of marginal zone B cells to antigen localization on follicular dendritic cells

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Summary. Injection of heat-killed *Escherichia coli* into rats results in massive loss of IgM + ve, IgD − ve B cells from the marginal zones of their spleen within 4 hr. This is matched by a concomitant increase of cells with this phenotype in the splenic follicles. The marginal zone remains depleted and the follicles distended for about 16 hr, but the histological picture returns to normal within 24 hr. Surface marker analysis of blood and spleen B lymphocyte populations throughout the course of the migration suggest that there is intra-splenic migration of IgM + ve cells from marginal zone to follicles rather than via the circulation.

Factors inhibiting localization of immune complex on follicular dendritic cells were assessed for their influence on marginal-zone B cell migration. Immune complex, injected 5 hr post-endotoxin administration localized poorly on follicular dendritic cells. While C3 depletion, by cobra venom, has no effect on marginal-zone B cell migration induced by endotoxin, it completely inhibits transport of heat-aggregated human gammaglobulin to follicular dendritic cells.

Abbreviations: PBS, phosphate buffer saline; CVF, cobra venom factor; FITC, fluorescein isothiocyanate; HGG, human gammaglobulin; PALS, periarteriolar lymphocytic sheath.

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INTRODUCTION

Recirculating B lymphocytes migrate between follicles of secondary lymphoid organs (Nieuwenhuis & Keuning, 1974; Nieuwenhuis & Ford, 1976). Typically these recirculating small B cells express both IgM and IgD on their surface (Gray *et al.*, 1982). Studies in rats have shown that a distinct population of B lymphocytes, which do not recirculate, is found in the marginal zones of the spleen (Gray *et al.*, 1982; Kumararatne, MacLennan & Bazin, 1981; Bazin *et al.*, 1982). These cells have surface membrane IgM but not IgD. Morphologically they are larger cells than surface IgD + ve follicular B cells, they have less condensed chromatin and differ in their receptors for the third component of complement (Gray *et al.*, 1984).

Although procedures which deplete recirculating B cells do not reduce marginal-zone cell numbers (Gray *et al.*, 1982; Kumararatne *et al.*, 1981), it has been known for many years that temporary depletion of marginal zones follows injection of Gram-negative bacterial vaccines. This was first noted by Petterson, Borgen & Graupner (1967), who induced the effect with heat-killed *Escherichia coli* and with paratyphoid vaccine. They also recorded that there was a concomitant accummulation of cells in the splenic follicles, which showed the morphological features of cells normally found in the marginal zones. These observations have subsequently been confirmed by several other groups using both killed Gram-negative bac-

terial preparations (Abe & Ito, 1972; Veerman & van Ewijk, 1975; Veerman & de Vries, 1976) and purified endotoxin (Kool *et al.*, 1967).

In parallel studies, Van Rooijen (1973, 1975) found that injection of paratyphoid vaccine abolished localization of immune complexes in germinal centres. After intravenous injection of immune complex (Van Rooijen 1973, 1977) or aggregated gammaglobulin (Brown *et al.*, 1973) initial splenic localization is in the primary blood sinusoids, which perfuse the marginal zone. This localization is on the surface of marginal-zone lymphocytes as well as macrophages (Mitchell & Abbot, 1971; Veerman & Van Rooijen, 1975). At about 2 hr after injection, complexes are found in the lymphocyte-rich corona of follicles, but by 24 hr the complexes are only found on dendritic cells of germinal centres (Van Rooijen, 1977). Brown *et al.* (1973) suggested that marginal-zone lymphocytes might be responsible for the transport of complexes to germinal centres. The object of the present study has been to re-investigate lymphocyte depletion from the marginal zone, induced by Gram-negative bacteria. In this, we have been able to use immunohistological markers, which identify the distinct phenotype of marginal-zone B cells. We have also investigated the relevance of this phenomenon to localization of immune complexes on follicular dendritic cells.

MATERIALS AND METHODS

Animals

(Lou × DA) Fl hybrid rats derived from highly inbred parental strains were used in the experiments described in this communication. These animals were bred and maintained under standard laboratory conditions in the animal house of the Department of Immunology, University of Birmingham. Within any one experiment animals were age and sex matched.

Antisera

Rabbit antibodies to rat IgM, IgD, IgG_{2c} and IgA were kindly provided by Dr H. Bazin, University of Louvain, Brussels. These antisera were prepared and their specificity tested as described by Bazin, Beckers & Querinjean (1974) and Bazin *et al.* (1978). Swine-anti-rabbit Ig and rabbit peroxidase-anti-peroxidase (PAP) used as second and third layers of immunoperoxidase staining were purchased from Dakopatts, Copenhagen, Denmark.

Endotoxin treatment

Escherichia coli (NCTC strain 9111 Stoke W 0111K58 (B4) H) were grown in nutrient broth overnight at 37°C, they were centrifuged and washed twice in phosphate-buffered saline (PBS), after which they were heat-killed by placing at 100°C for 15 min. The number of bacteria in the suspension, immediately before heating, was determined by counting colonies growing from a dilution of this suspension, plated on to nutrient agar. The final suspension for injection was $1{\cdot}8 \times 10^{10}$/ml. Rats were given intravenous (i.v.) injections of 0·5 ml of this heat-killed *E. coli* suspension.

Decomplementation

Rats were treated with 50 μg of purified cobra-venom factor (CVF) (Sigma, Poole, U.K.) in saline. This was given in two intraperitoneal (i.p.) injections of 25 μg, spaced by 24 hr. These animals were used in experiments 2 days later. This protocol was adapted from techniques described by Pryjma & Humphrey (1975). Animals were bled before and after treatment in order to assess the degree of decomplementation, as described below.

Serum complement levels were measured using an adaptation of a CH50 assay described by Hudson & Hay (1980). An IgG fraction of rabbit anti-ox red cells antibody, donated by Dr N.R. Ling, Department of Immunology, University of Birmingham was used to sensitize ox red cells. Fifty μl of a 1% suspension of sensitized red cells was added to each well of a microtitre tray, in which doubling dilutions of test sera had been made. Control wells containing no serum or 2% acetic acid were also set up. After incubation at 37° for 30 mins the trays were spun. The 50% lysis point was assessed by spectrophotometric measurement of assay supernatants.

Germinal-centre localization of aggregated human gammaglobulin.

The process of localization of aggregated gammaglobulin was demonstrated by the iv. injection of 1 mg of fluorescein-labelled heat-aggregated human gammaglobulin (FITC-HGG) 24–48 hours prior to sacrifice. FITC-labelled heat-aggregated HGG was kindly provided by Professor J. H. Humphrey, Royal Postgraduate Medical School, London. At sacrifice spleens were snap frozen and 5 μm sections cut on a cryostat. These sections were fixed in cold methanol (5 min) and then mounted in buffered glycerol containing 25 g/l 1, 4-diazobicyclo-(2,2,2)-octane (DABCO) to prevent fading (Johnson *et al.*, 1982).

Immunohistology

Surface membrane immunoglobulin was detected on acetone-fixed cryostat sections using an immunoperoxidase technique that is fully described in Gray *et al.* (1982).

Lymphocyte surface marker analysis

Spleen-cell suspensions were made by pressing a weighed portion between artery forceps, in a Petri dish, while immersed in tissue culture medium (RPMI 1640) containing 2% fetal calf serum (FCS). This suspension was taken up through a 21G needle and the cells then washed twice. Blood lymphocytes were separated on 9% Ficoll-Triosil. Blood was diluted 1 in 2 with RPMI, layered onto 9% Ficoll-Triosil and spun at $500 \times g$ for 20 min. The mononuclear cells at the interface were taken off and washed twice.

These washed cells were then evaluated using a double rosette assay to analyse their surface membrane IgM and IgD expression. Details of this double rosette assay, fluorescein and rhodamine labelling of ox red cells and the coating of these red cells with antibody is contained in Gray *et al.* (1982)

RESULTS

Immunohistological investigation of the changes occurring in the splenic B cell compartments following injection of heat-killed *E. coli*

Animals were injected i.v. with 0·5 ml of a suspension of $1{\cdot}8 \times 10^{10}$ heat-killed *E. coli*/ml. Groups of animals were killed at various times after injection. The spleens of these animals were examined on frozen sections using immunoperoxidase staining to reveal surface membrane immunoglobulin. The serial sections of the spleens from animals sacrificed 1 and 2 hr after injection appeared normal, when stained to reveal, respectively surface membrane IgM and IgD (Fig. 1a, b). After 4 hr, however, there was a loss of IgM+ve IgD−ve cells from the marginal zones. The few remaining cells were located adjacent to the marginal sinus. These IgM+ve B cells occupied less than one-half of the width of the marginal zone (Fig. 2a) and in places were lost completely. At the same time, the follicles appeared to be markedly enlarged. This enlargement was associated with the appearance of large numbers of IgM+ve IgD−ve cells. Staining for surface IgD (Fig. 2b) gave the impression that the follicles were depleted of IgD+ve cells. However, this effect may have been caused by a dilution of IgD+ve cells by the IgM+ve IgD−ve cells, which had entered this area. In many follicles at 4 hr the outer rim consisted almost exclusively of IgM+ve cells (Fig. 2b), suggesting that this may be their point of entry. Actual movement of IgD+ve cells to and/or from the follicles is suggested by the presence of large numbers of IgD+ve cells in the traffic zones in the outer regions of the periarteriolar lymphocytic sheath (PALS).

Six hr post-injection, the marginal zones were further depleted and in places the band of IgM+ve cells was completely lost. The follicles were more distended with IgM+ve IgD−ve cells. The distribution of cells at 8, 10 and 12 hr, after injection, was essentially the same, although in some animals at 12 hr, the band of cells in the marginal zone appeared less broad than it had been at 4 or 6 hr. By 16 hr repopulation of the marginal zone had commenced, IgM+ve IgD−ve cells were again found in the region adjacent to the marginal sinus. While follicles were still enlarged, the proportion of IgD+ve cells had risen, although it was not yet back to normal. The presence of large numbers of IgD+ve cells in the traffic zones was particularly noticeable at this stage. Twenty-four hr after injection of *E. coli* the white pulp morphology had returned to normal.

Previous work describing this phenomenon, using conventional haematoxylin and eosin (H+E) staining, noted the loss of morphologically identifiable germinal centres (Veerman & de Vries, 1976). In this study it was found that although germinal centres became difficult to distinguish, they were still present as evidenced by characteristic IgM-containing complexes on follicular dendritic cells (Fig. 2a). It seems likely that germinal centres became less distinct because of the entry of IgM+ve IgD−ve cells into their outer regions.

A proportion of marginal-zone B cells express IgA and/or IgG_{2c}, in addition to IgM, on their surface (Bazin *et al.*, 1982). Immunohistological analysis using these markers showed that an equivalent proportion of IgM+ve IgD−ve cells found in the follicles after *E. coli* injection carried these isotypes.

Is the migration of IgM+ve IgD−ve cells to follicles via the blood or by an intrasplenic route?

This question was addressed by determining the proportions of IgM+ve IgD−ve and IgM+ve IgD−ve B cell populations in the blood and spleen throughout the period following endotoxin-induced

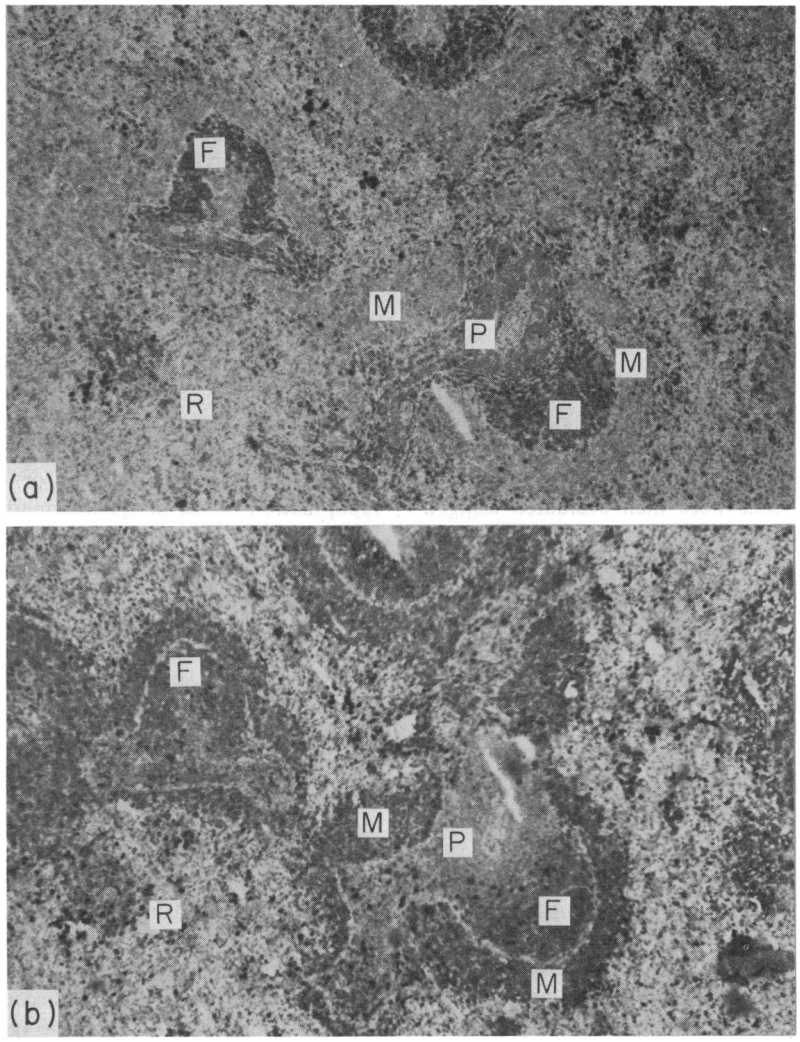

Fig. 1. Serial frozen sections of spleen from a normal adult rat (×13) (a) Stained to reveal surface membrane IgM note the normal width of the marginal zones (M) and the size of follicle (F). P = PALS; R = red pulp; G = germinal centre. (b) Stained for surface membrane IgD, note that the majority of cells in the follicle (F) are positive.

migration. This information was obtained by double rosetting of blood and spleen lymphocyte suspensions at various times following a single injection of heat-killed *E. coli*. Groups of six treated rats and one control were killed at each stage.

There was a the fall in blood lymphocyte numbers 4 hr after injection, this low count was maintained until 16 hr (legend to Fig. 3). Figure 3 shows that the proportion of IgM+ve IgD−ve and IgM+ve IgD+ve B cells, in the blood, was not altered during this time. There was no increase of the IgM+ve IgD−ve population, as might be expected if the marginal-zone B cell migration occurred via the blood. The decrease in blood lymphocyte numbers was concurrent with an increase in spleen lymphocyte counts after 4 hr (Fig. 4), however, the proportions of IgM+ve IgD−ve and IgM+ve IgD+veB cell subsets in the spleen were unchanged. Although there were increases in all populations 4 hr after injection of *E. coli*, these increases were small.

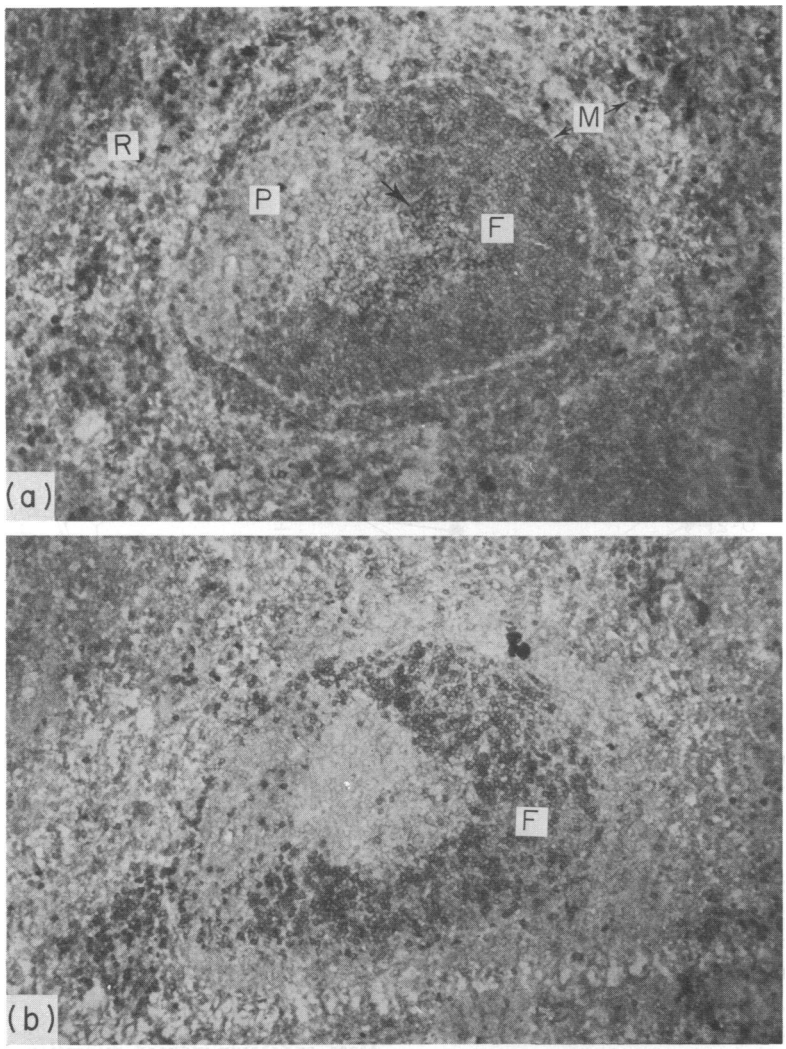

Fig. 2. Serial frozen sections of spleen from a rat injected 6 hr previously wth heat-killed *E. coli*, showing detail of a follicle photographed at × 325. (a) Stained for surface membrane IgM, the +ve cells now only occupy the inner party of the marginal zone (M). Follicles (F) are increased in size. Follicular dendritic cells are identified by IgM-containing immune complex (arrowed). P = PALS; R = red pulp. (b) Stained for surface membrane IgD, the follicle can be seen to contain an increased proportion of IgM+ve IgD−ve cells compared to normal. The outer region of the follicle contains a larger proportion of IgM+ve IgD−ve cells than the centre.

Is the endotoxin-induced migration of marginal-zone B cells complement-dependent?

Germinal-centre formation is complement-dependent (White *et al.*, 1975; Klaus & Humphrey, 1977); Papamichail *et al.*, 1975). Marginal-zone B cells have avid receptors for C3b, C3bi and C3d (Gray *et al.*, 1984). Also, endotoxin is a potent activator of the alternative complement pathway. These points led us to investigate the possibility that marginal-zone B cells are stimulated to migrate following binding to one or more of their C3 receptors. Animals were injected with heat-killed *E. coli* 2 days after the last injection of cobra-venom factor (CVF) (Pryjma & Humphrey, 1975). Bleeds were taken before and after CVF treatment and assayed for complement levels. All of the animals used were found to be completely decom-

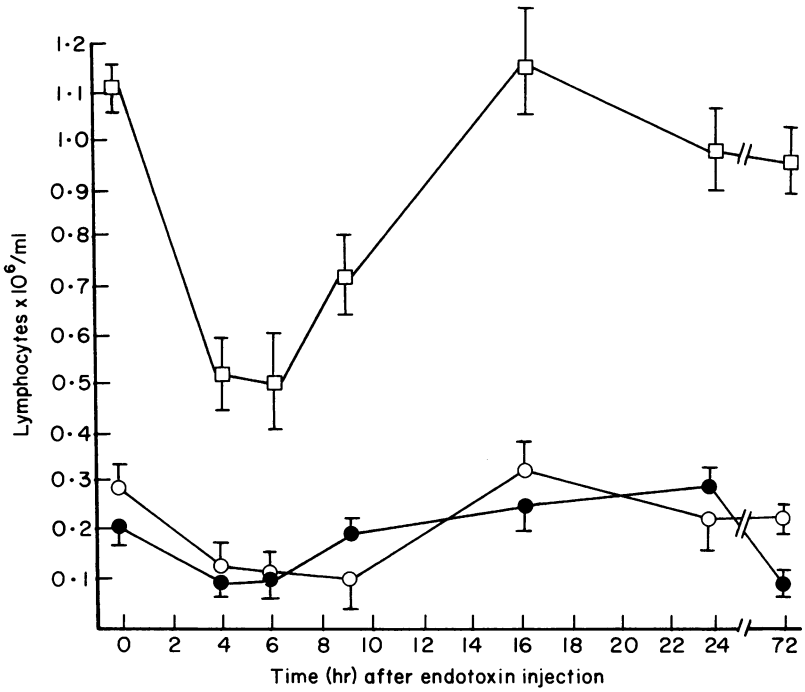

Fig. 3. Changes in B lymphocyte subpopulations in the blood, after injection of heat-killed *E. coli*. IgM + ve IgD − ve (●——●), IgM + ve IgD + ve (□——□) and IgM − ve IgD + ve (○——○) cell subsets were analysed using a double rosette assay. Each point represents the mean (± SE) of results from six rats. Total lymphocyte counts (× 10^6/ml) were, 6·5 at time 0, 3·4 at 4 hr, 3·0 at 6 hr, 3·2 at 9 hr, 6·6 at 16 hr, 5·2 at 24 hr and 5·2 at 72 hr.

plemented, as assessed by the inability of their sera to support lysis of sensitized red cells at a 1 in 2 dilution. Before CVF treatment these animals had CH50 titres > 1 in 80. Two animals were sacrificed at 4, 6 and 8 hours after the *E. coli* injection and spleens examined immunohistologically. In decomplemented rats, 4 hr post-injection, migration of marginal-zone B cells into follicles was shown to have occurred, as evidenced by staining of surface membrane IgM and IgD (Fig. 5a, b). The picture at 6 and 8 hr after *E. coli* injection also indicated that the migration had occurred in the spleens of these animals. In a second experiment analysis of the spleens from five more CVF-treated animals, removed 5 hr after injection of heat-killed *E. coli*, showed similar depletion of marginal zones and influx of IgM + ve IgD − ve cells into follicles.

Included in the protocol of this second experiment was the i.v. injection of FITC-labelled heat-aggregated HGG into CVF-treated rats to see if the degree of decomplementation achieved was sufficient to abolish localization of complexes in germinal centres. While a group of five normal rats exhibited good germinal-centre localization, no FITC-labelled HGG was observed on follicular dendritic cells in the spleens of the five CVF-treated rats.

Does endotoxin-induced migration of cells from the marginal zone inhibit the localization of FITC-HGG in germinal centres?

Reports of Van Rooijen (1973, 1975) describe the abolition of germinal-centre localization of immune complexes following an injection of paratyphoid vaccine. This is consistent with the hypothesis that marginal-zone B cells transport complexes into follicles. In this study a group of five rats were given an injection of FITC-labelled heat-aggregated HGG 5 hr after an injection of *E. coli*, i.e. at a time when the marginal zone shows maximum depletion. These animals were sacrificed 24 hr later and the localization was observed in frozen spleens. Two rats exhibited no germinal-centre localization, two showed good locali-

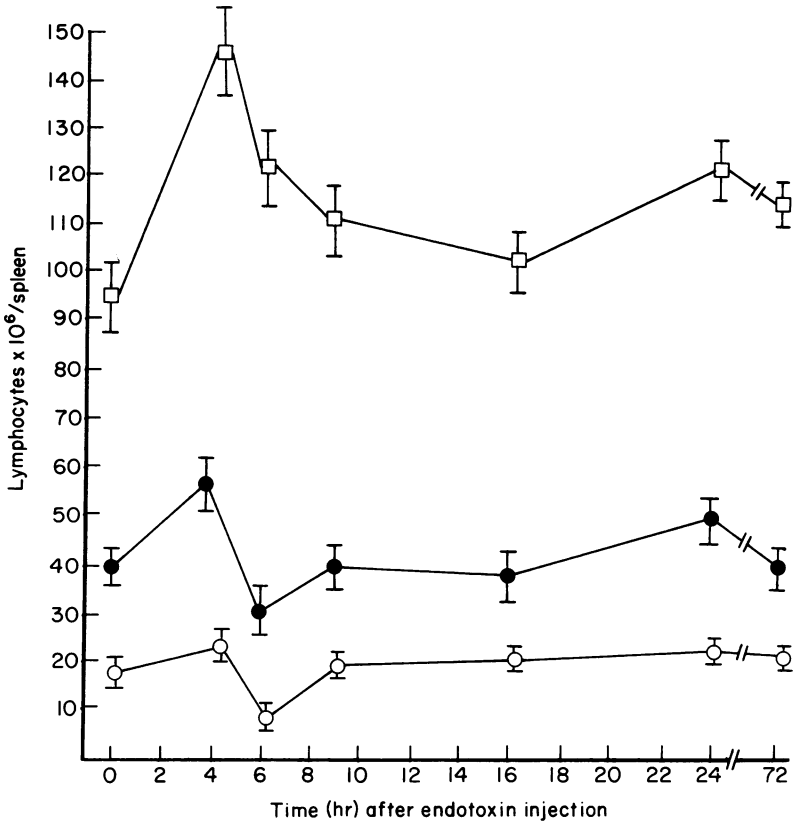

Fig. 4. Changes in B lymphocyte subpopulations in the spleen, after an injection of heat-killed *E. coli*. IgM+ve IgD−ve (●——●), IgM+ve IgD+ve (□——□) and IgM−ve IgD+ve (○——○) cell subsets were analysed, in spleen-cell suspensions using a double rosette assay. Each point represents the mean (±SE) of results from six rats. Number of lymphocytes per spleen ($\times 10^6$) were, 302 at time 0, 454 at 4 hr, 382 at 6 hr, 358 at 9 hr, 349 at 16 hr, 421 at 24 hr and 369 at 72 hr.

zation while in the fifth rat only very sparse localization of complex was observed.

DISCUSSION

The experiments reported in this paper using objective markers for marginal-zone B cells confirm the observations of Pettersen *et al.* (1967), Veerman & Van Ewijk (1975) and Veerman & de Vries (1976) that there is migration of marginal-zone B cells into follicles following a single injection of heat-killed *E. coli*. We find that the maximum depletion of IgM+ve IgD−ve cells in the marginal zone occurs 4–6 hr after injection of *E. coli* and that this coincides with a gross enlargement of follicles associated with the arrival IgM+ve, IgD−ve cells. There are variations between studies in the timescale of this migration, however, these may be explained by differences in dose, type of vaccine and strain of rat used.

Surface marker analysis of spleen and blood lymphocyte suspensions at various times after injection of *E. coli* suggests that the migration of marginal-zone cells into follicles occurs via an intra-splenic route and not via the circulation. It seems reasonable to assume that the pathway of this migration is along the traffic corridor in the outer region of the PALS, as there are large numbers of cells in this area during the period of migration. The observation of a predominantly IgM+ve IgD−ve outer rim of cells in the follicles, at the height of the migration, suggests that IgM+ve IgD−ve cells enter the follicles from the traffic zones in this region. The apparent follicular IgM+ve cell depletion is in part due to a dilution effect of the

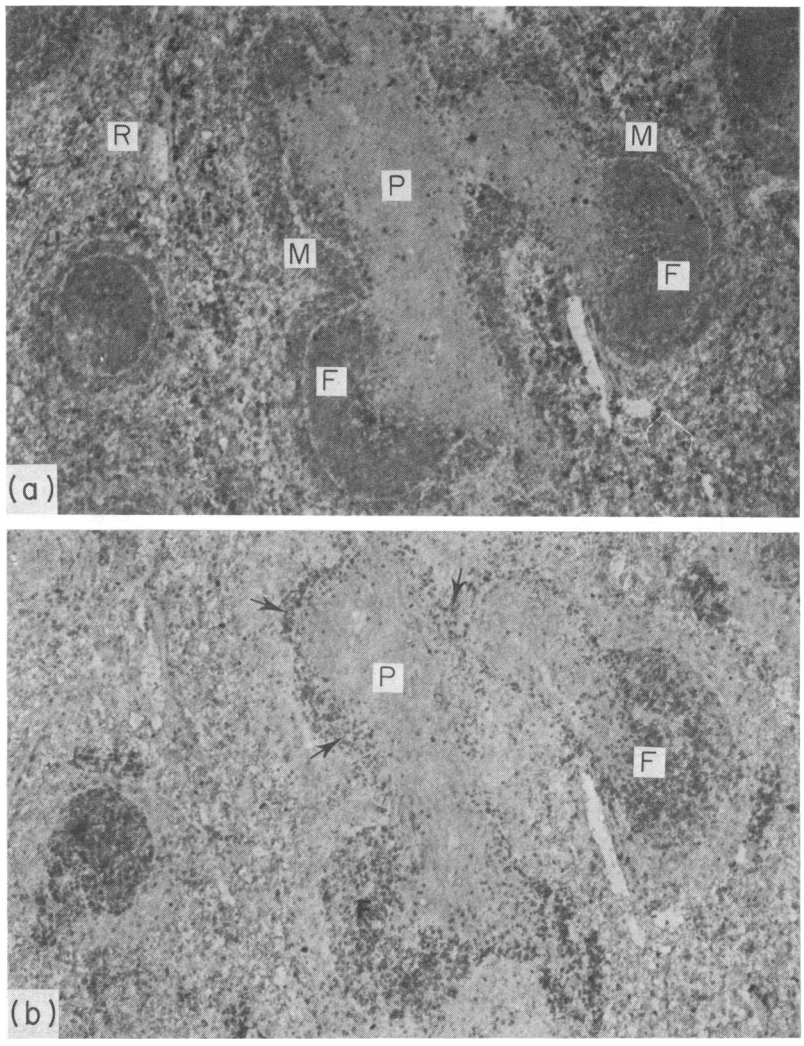

Fig. 5. Serial frozen sections of spleen (× 130) from a decomplemented rat, injected 4 hr previously, with heat-killed *E. coli*. (a) Staining for surface membrane IgM shows that the marginal zone (M) is depleted of B cells, compared to controls. Follicular (F) enlargement is also evident. (b) Staining for surface membrane IgD. There are large numbers of IgD − ve cells in follicles (F). Arrows indicate the many IgD + ve cells in the traffic zones on the outer aspect of the PALS (P).

invading IgM + ve IgD − ve cells. However, this may be accentuated by emigration of IgD + ve cells, for the large numbers of IgD + ve cells are found in the traffic zone on the outer edge of the PALS. LPS stimulation, *in vitro* is known to cause a loss of smIgD from B cells over a 72-hr period (Kearney & Abney, 1978). The loss of smIgD positivity in follicles following endotoxin administration occurs much more rapidly than this. Also the proportion of IgM + ve cells in the spleen is unaltered following endotoxin administration.

Migration of IgD + ve cells from follicles may not be the only cause of large numbers of such cells in the traffic zones. Surface marker analysis of blood lymphocytes shows that within 4 hr of *E. coli* injection there is a fall in B cell numbers lasting until about 9 hr. Concurrent with this is an increase in the B lymphocyte numbers, of each subset, in the spleen. This phenomenon is reminiscent of the non-specific cell shutdown seen in single cannulated lymph nodes when challenged with various antigens (Hall & Morris,

1965; Smith & Morris, 1970; Hay, Lachman & Trnka, 1973; Cahill, Frost & Trnka, 1976). The sequestration of recirculating antigen-specific cells in the spleen following i.v. administration of antigen is well-documented (Sprent & Lefkovits, 1976; Ford, Simmonds & Atkins, 1975; Ford, 1972). However, in only one of these studies (Ford, 1972), is a non-specific retention of recirculating cells in the spleen reported. In this case administration of tetanus toxoid caused transient accummulation of both antigen-specific and antigen-non-specific cells in the spleen. Also, Ford (1969) reported an inhibition of the release of cells from a perfused spleen when sheep red cells were infused. The *E. coli* preparation used in our experiments may elicit a similar effect.

The return of splenic compartments to normal within 24 hr may result from reverse migration of marginal-zone B cells to their original position. It is also possible that IgM + ve IgD − ve cells, upon reaching follicles, acquire smIgD and remain there, while the marginal zone is repopulated with cells newly generated from precursors.

Demonstration that marginal-zone B cells can be induced by Gram-negative bacterial endotoxins to migrate into follicles is also of interest because these cells may transport immune complexes into germinal centres. After injection of immune complex or antigen into primed or unprimed animals we have not seen large scale migration of marginal-zone B cells into follicles as occurs after an injection of endotoxin. However, it is possible that small numbers of cells, after binding antigen or antigen-antibody complex, do travel into follicles. Veerman & de Vries (1976), in studying the histological changes following immunization with sheep red blood cells noted no gross splenic histological changes but did observe small numbers of medium-sized cells in the periphery of the follicles. They noted a similarity between these medium-sized lymphocytes and marginal-zone cells.

Cellular transport of immune complexes is likely to involve binding via antigen-specific receptors or receptors for Fc or C3 or LPS. Our experiments with decomplemented rats, in agreement with the work in the mouse (Klaus & Humphrey, 1977; Papamichail *et al.*, 1975) and in chickens (White *et al.*, 1975) show the loss of capacity to localize aggregated-HGG in germinal centres. However, C3-depletion has no effect on endotoxin-induced migration of marginal-zone B cells. Thus, the marginal-zone B cell migration, induced by endotoxin, is not mediated by binding C3 components to their receptors. Thus while C3 receptors on marginal-zone cells may induce migration, they are not necessary for its induction. Endotoxin is a polyclonal B cell activator (Peavy, Adler & Smith, 1970; Gery, Kruger & Spiesel, 1972) and binding to LPS/endotoxin receptors may be involved in stimulating B cells to migrate. Experiments by Wallis & Chaudhuri (1982), in mice, suggest that marginal-zone cells may be preferentially activated by this agent. The migration stimulus may be provided indirectly via interaction with macrophages, as endotoxin has been shown to induce macrophages to secrete factors that activate B lymphocytes (Morrison & Ryan, 1979; Wood *et al.*, 1976).

The outcome of the experiment to investigate localization in germinal centres of fluorescein-labelled aggregated-HGG injected 5 hr after a dose of *E. coli* proved inconclusive. Van Rooijen (1975) showed that the administration of paratyphoid vaccine, before injection of complex, inhibited the localization of the complex in germinal centres. We were only able to see such inhibition in a proportion of animals. The timing of the injection of aggregated-HGG, in relation to the administration of endotoxin may be important and a fuller investigation along these lines is required. Both Van Rooijen (1975) and Pierce (1966) have demonstrated that endotoxin given at the same time as labelled complex or antigen (in primed animals) is associated with increased uptake of labelled material on follicular dendritic cells. These data are not inconsistent with the hypothesis that marginal-zone B cells may be involved in the transport of immune complexes into follicle centres. However, direct evidence in support of this mechanism is still lacking.

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